

ABO and Rh Hemolytic Disease of the Fetus and Newborn

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Abstract: - Hemolytic disease of the fetus and newborn (HDFN) is a blood disorder in which red blood cells are destroyed faster, leading to anemia. Our study focused on immune-mediated HDFN. The study included 86 newborns with HDFN. Samples were provided to two clinics in Georgia. Coombs tests were performed to confirm the presence of HDFN. Monoclonal antibodies were used to determine ABO and Rh blood group antigens. The genetic analysis was performed using the SNP rs8176719. ABO-HDFN was the majority, followed by Rh-HDFN ($\chi^2 = 6.44$, $p = 0.04$). the majority newborns with ABO-HDFN have A(II), Rh+ blood group ($\chi^2 = 141.16$, $p < 0.001$). A(II), Rh+ blood group, found in $50.00 \pm 11.1\%$ of cases of Rh-HDFN, followed by O(I), Rh+ blood ($\chi^2 = 52.80$, $p < 0.001$). 7 samples carry a complete deletion (-;-). It is a homozygous OO genotype. 27 newborns with HDFN show the heterozygosity (-;G) condition (AO and BO genotypes) ($\chi^2 = 34.6$, $p > 0.001$). The majority of cases of ABO-HDFN involve an O blood group mother/A blood group newborn. In Rh-HDFN cases, most of it was Mother O(I), Rh- /newborn O(I), Rh+. The complete deletion of c.261delG in HDFN is relatively rare. The presence of the (-; G) genotype in HDFN with non-O groups reduces antigen expression, since these genotypes are AO or BO, thereby reducing the frequency of ABO-HDFN. In ABO-HDFN, the highest percentage is the O/A phenotype, which is the maternal/newborn phenotype. In the case of Rh incompatibility, it is Rh+ vs. Rh-.

Key-Words:- newborns, Hemolytic anemia, blood, ABO and Rh incompatibility, rs8176719, genetics.

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1 Introduction

HDFN is one of the most common blood disorders in which red blood cells (RBCs) are destroyed faster than the bone marrow can replace them. Based on this alteration, the blood profile indicates anemia. There are many causes of HDFN. The most common are: bone marrow failure, autoimmune problems, hereditary blood diseases, side effects of some drugs, various types of infections, complications of blood transfusion, immunocompromised pregnancy, and infantile pyknocytosis [1], [2], [3], [4], [5], [6].

Our study of interest was the immune-mediated

HDFN. Incompatibility in the ABO blood group system represents the most frequent etiology, followed by discrepancies in the Rh and Kell systems [7], [8], [9]. HDFN due to anti-K antibodies tends to be severe, even at low titers [10]. The Kidd antigen system is frequently implicated in delayed hemolytic reactions [11]. Duffy and MNS antigens are considered to have moderate clinical relevance. There are additional blood group immunizations as well [12].

ABO-HDFN is generally less severe than Rh-HDFN. ABO-HDFN commonly contributes to neonatal hyperbilirubinemia, particularly in A or B

blood group newborns born to O blood group mothers [13]. ABH antigens (A, B, H) are carbohydrate-based epitopes, which are presented on the cell surface of various tissues, including RBCs [14]. In most cases, individuals have natural IgM antibodies that can bind to A and B antigens and, in the event of incompatibility, trigger hemolysis by activating the complement system. Pregnancy or transfusion can elicit IgG antibodies that cross the placenta, thereby elevating the risk of HDFN. Erythrocyte damage starts during pregnancy, but is clinically manifested postnatally, including blood agglutination, hyperbilirubinemia, anemia, and other complications.

ABO-HDFN cannot always be prevented. Understanding the ABO_HDFN pathophysiology enables strategies to mitigate incidence and sequelae. Rh-HDFN occurs when an RhD-negative mother develops immune antibodies against a fetal RhD⁺ erythrocyte. This process commences with maternal sensitization, followed by transplacental passage of IgG antibodies, culminating in HDFN. Although the RhD antigen is the most immunogenic, the Rhc antigen also provokes substantial alloimmunization, potentially resulting in severe HDFN [15]. Anti-D immunoglobulin prophylaxis has substantially reduced the incidence of HDFN in high-resource regions. However, low-resource areas continue to report elevated rates, understanding the urgent need for universal access to anti-D immunoglobulin among Rh-negative pregnant women [16], [17], [18].

RBC antigens were usually studied serologically. Advances in molecular technologies now enable genetic testing, which provides reliable data on the genes that encode group antigens - a critical tool for newborns with a severe HDFN. Newborns have reduced expression of blood group antigens [19], rendering serological typing unreliable. Therefore, in cases where hemotransfusion therapy is necessary, genetic analysis is especially important.

The goal of our research is to study the c.261delG via SNP rs8176719. SNP rs8176719 is the primary SNP used to determine O blood group status. No prior document has HDFN associations with SNP rs8176719 in newborns, despite its study in diverse populations for links to disease such as venous thromboembolism [20], [21], [22], ischemic stroke [23], heparin-induced thrombocytopenia [24], and COVID-19 infection [25]. We consider routine clinical implementation of this genotyping methodology necessary, particularly for severe HDFN cases requiring urgent hemotransfusion.

2 Research Materials and Methods

The biological material was obtained from newborns with HDFN. Blood samples were provided by two clinics in western Georgia: "Iris Borchashvili Health Center, Medina" and "M. Iashvili Batumi Central Hospital for Mothers and Children" (Batumi). This was an observational cohort study. The study was conducted in accordance with relevant ethical standards and was approved by the Research Ethics Committee (N BIH-24-0215-01). The qualitative and genetic characteristics of group antigen-antibody systems were analyzed at the "A. Diasamidze Immunogenetics" and "Biosafety Laboratory" located in the Batumi Shota Rustaveli State University (BSU).

The study included 86 newborns with HDFN. The degree of hemolysis was assessed using laboratory parameters, such as total serum bilirubin. Direct and indirect Coombs tests were performed to confirm immune-mediated HDFN. ABO and Rh blood groups were determined by monoclonal anti-A, anti-B, and anti-D antibodies. The majority of cases (N=59) were due to ABO-HDFN and Rh-HDFN, but in some cases (N=27), unspecified HDFN was also observed.

Genetic analysis was performed in a subset of 34 newborns with Immune HDFN. The reduced number of samples available for genetic testing was due to the use of residual blood remaining after routine clinical laboratory testing. We did not perform additional invasive procedures to obtain the newborn's blood and used leftover blood from the clinic's laboratory instead.

We used the single-nucleotide polymorphism (SNP) rs8176719 during the study. The mentioned SNPs have the potential to detect a 261G deletion in exon 6, thereby enabling detection of the ABO system O allele (in samples homozygous for the deletion). In samples where the complete deletion is not detected in the homozygous condition, the individual belongs to the non-O group (A, B, or AB). Individuals homozygous for the deletion (OO genotype) are classified as blood group O, whereas samples without the homozygous deletion belong to the non-O blood groups (A, B, AB). In addition, heterozygous carriers of the O allele (AO or BO) can be identified. Because only rs8176719 was analyzed, this assay does not provide complete ABO genotyping and cannot distinguish all ABO genotypes or allelic variants.

Genotyping of the ABO261G deletion was performed by the polymerase chain reaction (PCR) method. We used 5'CCTGTGTGGATGTGCAGTAGGA3',

5'CGTTGAGGATGTCGATGTTGAA3' primers and Fam-TCCTCGTGGTGACCCCTTGGC-TAMRA, Vic-ATGTCCTCGTGGTACCCCTTGGCT-TAMRA probes. DNA extraction from each sample was performed using an automated KINGFISHER DUO extractor with MagMax™-96 DNA Multi-Sample Kit (Applied Biosystems, Vilnius, Lithuania).

All assays were performed using a 96-well Real-time PCR system with the appropriate QuantStudio 5 software. Amplification reactions were performed in duplicate at 10 ng DNA concentration (DNA was diluted with PCR water and quantified using a NanoDrop/Thermo Scientific NanoDrop lite Spectrophotometer), TaqMan Universal Master Mix buffer (Applied Biosystems), and thermal cycling with the following conditions: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and (Annealing/Extension) annealing/chain extension at 60°C for 60 s. The obtained results were interpreted using various plots.

Statistical analysis was performed using standard biostatistical methods. For categorical variables, frequencies, percentages, and 95% confidence intervals calculated using the Wilson score method were reported. Differences between categorical variables were evaluated using the chi-square (χ^2) test. The χ^2 goodness-of-fit test was conducted to assess whether observed genotype or phenotype frequencies differed significantly from the expected distribution. [26] was used for statistical analysis.

3 Results

Our study group included 86 newborns with HDFN. The majority of cases (N=59) were due to ABO-HDFN and Rh-HDFN, but in some cases (N=27), unspecified HDFN was also observed. ABO-HDFN was caused by 45.35% (N=39). Rh-HDFN was 23.26% (N=20). 31.40% of the cases were unspecified-HDFN (N=27) (Table 1, Appendix). The distribution of HDFN types differed significantly from the expected distribution ($\chi^2 = 6.44$, $df = 2$, $p = 0.04$), with a small-to-moderate effect size (Cohen's $w = 0.27$).

For the next stage of the study, we excluded cases of unspecified HDFN (n=27). We were interested in how erythrocyte phenotypic combinations were observed only in ABO-HDFN and Rh-HDFN cases.

Eight ABO/ Rh blood group combinations were studied in newborns with ABO-HDFN. Only 5 combinations were found in the target group.

Newborns with O(I), Rh+, O(I), Rh- blood group and AB(IV), Rh- blood group are not found in the mentioned newborns (N=0). In this research group, the newborns have the highest rate of A(II), Rh+ blood group combination, 74.36% (N=29), followed by B(III), Rh+ blood group, 15.38% (N=6), followed by A(II), Rh- blood group, 5.13% (N=2). Newborns with AB(IV), Rh+ blood group and B(III), Rh- blood group have been reported in only one case, accounting for 2.56% of the research group (N=1) (Table 2, Appendix). The distribution of ABO/Rh combinations with HDFN newborns differed significantly from the expected distribution ($\chi^2 = 142.13$, $df = 7$, $p < 0.001$), with a very large effect size (Cohen's $w = 1.91$).

ABO/ Rh phenotypic combinations were analyzed in newborns with Rh-HDFN. They are distributed as follows: A(II), Rh+ blood group, found in 50.00% of cases (N=10), followed by O(I), Rh+ blood group, which equals 45.00% (N=9).

B(III), Rh+ blood group combination occurs in 5.00% (N=1) of the newborns. AB(IV), Rh+ blood group combination was not found (N=0). Of course, O(I), Rh-, A(II), Rh-, B(III), Rh-, and AB(IV), Rh- were not observed (Table 3, Appendix).

The distribution of ABO/Rh combinations with Rh-HDFN newborns differed significantly from the expected distribution ($\chi^2 = 36.80$, $df = 7$, $p < 0.001$) with a very large effect size (Cohen's $w = 1.36$). In the blood samples of 34 newborns with HDFN, one specific locus (c.261delG) was studied, which we identified using SNP rs8176719. SNP rs8176719 is the main SNP for determining O blood group status as per [27]. Results for SNP rs8176719 are interpreted as defined (Table 4).

Table 4. Interpreting genotypes and blood group using SNP rs8176719

Genotype	Analisis
(-;-)	O blood group
(-;G)	A and B blood groups
(G;G)	A, B, and AB blood groups

Source: created by the authors

An individual will usually have blood type O only if they have two copies of the deletion. They are genotyped for SNP rs8176719 (-;-). If they carry one copy (G;-), they may have type A or type B blood. A person with SNP rs8176719 (G;G) is likely to have blood type A, B, or AB (Table 4). The study does not allow distinguishing among the A, B, and AB blood groups. But, based on it, we can identify c.261delG, which characterizes the O

allele, which occurs in the homozygous state in the case of the O group. In the heterozygous state, we have one deleted area; accordingly, the A and B groups should be represented by the AO and BO genotypes, respectively. When the mentioned deletion does not occur, we can consider three genotypic states (AA, BB, and AB). The qPCR results are presented in various graphical plots. Two genotypic variants were identified in our study group. The first case is presented in the Figure 1.

There are two Fam-TCCTCGTGGTGACCCCTTGGC-TAMRA and Vic-ATGTCCTCGTGGTACCCCTTGGCT-TAMRA probes. When only one (Vic-prob) signal is visible, indicating a homozygous state (-;-), in which G is completely deleted; therefore, we have the O allele. The second case involves two signals (Vic and Fam), indicating a heterozygous (-; G) condition (Figure 2).

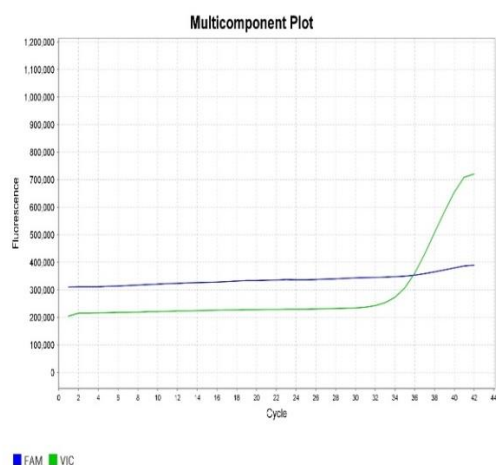


Fig. 1: Multicomponent plot for homozygous G deletion (-;-).

Source: Generated using QuantStudio™ 5 Real-Time PCR System.

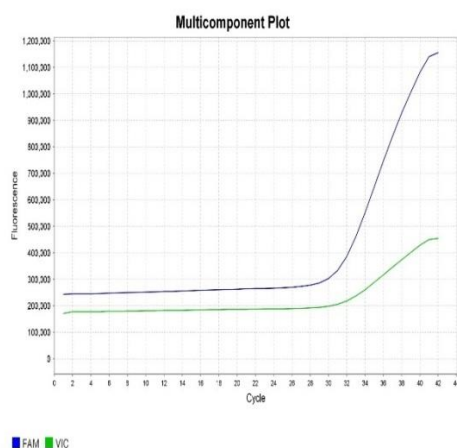


Fig. 2: Multicomponent plot for the heterozygous (-; G) case.

Source: Generated using QuantStudio™ 5 Real-Time PCR System.

Seven studied samples carry a complete deletion, in which the G nucleotide at position 261 is absent. A similar deletion is observed for alleles 1 and 2. According to the cited site, it is in a homozygous state (OO genotype); phenotypically, it is the O blood group. The 27 newborn samples show a signal on both probes. Allele 1 and allele 2 specificities are found. This indicates a heterozygote condition. One studied allele at position 261 has a G nucleotide deleted, and in the other allele, at the same position, we get a G nucleotide. That condition is (-; G). The AO and BO genotypes correspond to this variant (Table 5, Appendix).

The distribution of (-;-), (-;G) and (G;G) with HDFN newborns differed significantly from the expected distribution (χ^2 - 34.6, df=2, $p < 0.0001$) with a very large effect size (Cohen's $w = 1.01$).

As shown in the results, the prevalence of the O blood group is lower among the studied newborns with HDFN than among those with non-O blood groups. The prevalence of the G deletion in the studied samples was 100%, and the G nucleotide at the same locus was observed in 27 cases, representing $79.41 \pm 6.9\%$ of the samples.

We studied the blood group combination of the mother/newborn in ABO-HDFN. There are 7 combinations from the 64 possible combinations in the studied cases: 1. Mother O(I), Rh+ /newborn A(II), Rh+ (N=28); 2. Mother O(I), Rh+ /newborn A(II), Rh- (N=1); 3. Mother O(I), Rh+ /newborn B(III), Rh+ (N=6); 4. Mother O(I), Rh+ /newborn B(III), Rh- (N=1); 5. Mother O(I), Rh+ /newborn AB(IV), Rh+ (N=1); 6. Mother O(I), Rh- /newborn A(II), Rh+ (N=1); 7. Mother O(I), Rh- /newborn A(II), Rh- (N=1) (Table 6, Appendix). The majority of cases involve an O blood group mother with an A blood group newborn (N=31/39). This is followed by an O blood group mother with a B blood group newborn (N=7/39). There is only one case when an O blood group mother has an AB blood group newborn.

We also analyzed blood type combinations of the mother and newborn in cases of Rh-HDFN (N=20). In this case, only 4 out of 64 possible combinations were observed: 1. Mother O(I), Rh- /newborn O(I), Rh+ (N=9); 2. Mother O(I), Rh- /newborn A(II), Rh+ (N=3); 3. Mother A(II), Rh- /newborn A(II), Rh+ (N=7); 4. Mother B(III), Rh- /newborn B(III), Rh- (N=1). In any case, the mother is Rh- and the newborn is Rh+ (Table 7, Appendix).

4 Discussion

Our research results demonstrate that ABO incompatibility was the most frequent cause of HDFN, accounting for the majority of cases, whereas Rh incompatibility accounted for a smaller proportion. The most frequent combination in our study was O/A mother/newborn. The results of our study are consistent with the literature. A group of scientists studied 275 cases of HDFN in newborns caused by ABO antigens. Mothers with blood group O were the most prevalent in the study. The mother of 259 newborns was the carrier of this group. It is followed by 11 mothers of newborns with blood group B and 5 mothers of newborns with blood group A. Among newborns with A group, it was most common (151 cases), followed by group B (108 cases) and group AB (16 cases). The most frequent incompatibility was O/A, accounting for 54.9% of the studied cases. It was followed by O/B, B/AB, and A/AB maternal-fetal combinations [28]. Any articles refer to HDFN caused by ABO system incompatibility in the offspring of mothers with the O group. However, this does not mean that the offspring of a non-O blood-group mother are protected from HDFN. A very interesting and rare case has been reported in the literature: a group A newborn developed HDFN whose mother has the B blood group. IgG anti-A antibodies caused the disease. The direct Coombs test was negative, but the indirect Coombs test showed a strong positive reaction on standard A1 cells. It must be noted that the newborn had a high bilirubin level on blood analysis [29]. The above highlights the immunological complexity of maternal-fetal blood group incompatibility and emphasizes the importance of antibody screening in pregnant women regardless of ABO group.

The study reports 125 O RhD-positive pregnant women whose newborns had blood group A or B. In this study, the titer of anti-A antibody was significantly higher than that of anti-B antibody. This study also found that, when maternal antibody titers are high, a double-volume exchange transfusion is required. In contrast, for low titers, phototherapy is indicated [30].

It is also interesting to know which class of antibodies is synthesized in ABO-HDFN cases. In one research paper, 82 neonates with HDFN were studied. Immunoglobulin G1 was found in 22% of direct antibody titer-positive, group A and B blood group neonates born to O blood group mothers, whereas immunoglobulin G3 was rare. G1 was identified in 18 neonates, G3 was not identified in any neonate, and G2 and/or 4 were detected in 64 cases [13]. One scientific paper suggested that the

ABO-HDFN difference was due to maternal anti-A and anti-B antibodies. For predicting HDFN in A blood group newborns, maternal IgG1 subclass determination achieved an accuracy of 83% at birth. For B blood group newborns, an accuracy of 91% was achieved by the maternal IgG2 subclass determination. The authors note that, based on their study results, the A2 subgroup was not detected in newborns with HDFN, due to a quantitative deficiency of the A antigen in this subgroup [31].

It is important to determine the level of carboxyhemoglobin in the cord blood of newborns in the A or B group whose mothers have the O group. It is considered an indicator of HDFN and an early predictor of the need for phototherapy. The studied infants were divided into two groups: Group 1 included infants with blood group A or B whose mothers had O blood, and Group 2 included infants whose mothers did not have O blood. Total serum bilirubin and carboxyhemoglobin levels at birth were compared between the studied groups. Carboxyhemoglobin levels were higher in group 1 than in group 2. The treatment procedure: Phototherapy was initiated earlier in the first group than in the second. The study demonstrated increased carboxyhemoglobin levels in newborns with ABO incompatibility, and higher levels were associated with an earlier need for phototherapy [32].

Prevalence and frequency of anti-Rh antibodies vary across populations, which are significantly influenced by genetic and demographic factors. The research aim of the retrospective study in Sri Lanka, which involved 20,212 pregnant women and transfusion recipients, was to assess the prevalence, distribution, and clinical relevance of anti-erythrocyte antibodies. 0.80% of the study population was alloimmunized. Only 28.87% of antibodies were clinically significant. The study also revealed that the incidence of alloimmunization was significantly higher in pregnant women compared to transfused patients ($p < 0.000$). Among the D- pregnant women studied, 5.45% were alloimmunized. In some cases, HDFN was caused by anti-E antibodies [15].

In the majority of our Rh-HDFN cases, we do not have ABO incompatibility (17/20) (Table 7, Appendix). This is easily explained because natural hemolysis of ABO group incompatibility does not occur in this case, since the mother and the fetus have the same ABO blood group and the memory cells have time to recognize the Rh antigen, resulting in the synthesis of immunoglobulin G, which crosses the placenta and causes HDHF [33].

Rh-HDFN is now manageable and predictable. The introduction of IgG anti-D prophylaxis has substantially reduced the incidence and severity of RH-HDFN. The administration of anti-RhD immunoglobulin in RhD-negative pregnant women in the appropriate dose and at the appropriate time [33]. After the introduction of this procedure in Europe, the incidence of RhD-HDFN decreased by 85%. One recent study involving 370 pregnant women found an alloimmunization prevalence of 3.5%. The potential for severe HDFN associated with antibodies such as anti-K, anti-E, and anti-c necessitates vigilant monitoring at the primary care level [34]. The prevalence and trends of alloimmunization among 9,876,196 pregnant women in the United States of America from 2010 through 2021 were studied. Only 1.5% were positive for RBC antibodies. Of the identified anti-erythrocyte antibodies, the anti-D antibody was the most common. It was followed by anti-K antibody, and then anti-c antibody. Data analysis revealed that the incidence of these three high-risk antibodies is increasing from 2010 to 2021 ($P < .001$) [18].

There is little evidence to support the need for IgG anti-D prophylaxis in RhD-negative women undergoing first-trimester abortions. However, a meta-analysis of the literature suggests that guidelines need to be updated for Rh-negative women undergoing first-trimester abortions [35]. Studies have also shown that RhIG is a limited resource, and its intended goal is to reduce anti-D HDFN; more effective management of RhIG utilization is needed [36]. There are regions of the world where awareness and education levels regarding these issues are limited. Women are unaware of the risks involved, and preventive measures are also limited [37]. Accordingly, in these regions, it is necessary to establish focus groups and conduct training courses to develop women's risk awareness further and reduce the number of HDFNRh alloimmunization remains a critical problem in obstetrics, especially when RhD- pregnant women have not received RhIg prophylaxis after their first delivery. A 19-year-old woman, during her second pregnancy, had no documented use of anti-D prophylaxis after her first delivery, no history of spontaneous or induced abortions, ectopic pregnancies, or blood transfusions. The absence of the Kell antigen ruled out additional risks of alloimmunization. At 28 weeks of gestation, her indirect Coombs test was strongly positive with an anti-D antibody titer of 1:128. Further immunohematological evaluation revealed that the patient had blood group B with a

rare dCe/dce Rh phenotype. Although the fetus was at risk of HDFN, no intrauterine transfusion was required during pregnancy. This case highlights the importance of routine antenatal antibody screening, awareness of Rh phenotypes, and timely preventive measures to prevent Rh alloimmunization. Comprehensive monitoring and genotype-informed transfusion planning are particularly important in resource-limited settings [38].

As mentioned above, the SNP rs8176719 was used for genetic testing. Genetic research showed that only a small proportion of the studied samples carried a homozygous deletion (-;-), corresponding to the O blood group, while the majority were heterozygous (-;G), associated with the A or B phenotype. The study confirmed that homozygous O alleles are relatively rare in newborns with HDFN. In contrast, heterozygous alleles are more common, suggesting that ABO incompatibility in newborns with non-O blood groups may reduce the risk of or severity of HDFN. The results of this study indicate that SNP rs8176719 effectively identifies the c.261delG deletion and provides identification of the O allele, which is important for assessing the genetic basis of HDFN, despite the small cohort. 7 studied newborns with the O group were diagnosed with Rh-HDFN, which once again indicates that hemolysis caused by the ABO system incompatibility is not observed in O blood group newborns.

The results of our study were compared with the distribution of these markers in different groups. We studied the distribution of O and non-O blood groups in healthy newborns [19], 1-year-old infants [39], and blood donors [40], all previously studied in the region (Table 8, Appendix). As shown in the table, the distribution difference between the O and non-O groups is most striking in HDFN. This distribution is almost equal in newborns and one-year-old infants. A more similar distribution is found among blood donors, where the O and non-O groups are almost equally represented. The prevalence of the O group among newborns with hemolytic anemia is only 20.6%. The corresponding non-O blood group is 79.4%. χ^2 equals 14.7138 for df 3, $p = 0.002078$.

In parallel with the development of modern medicine, the management of HDFN is undergoing successful renewal, [10], [41], with planning and implementing early preventive measures. Treatment guidelines are also changing accordingly [42], [43], [44]. Early determination of the RhD status of the fetus is becoming particularly relevant and is being intensively used, e.g., with cfDNA

screening methods. Utilization of cfDNA screening for fetal RhD and other RBC antigen status is likely to increase in routine care [45], [46]. An interesting approach is also the dual-biomarker strategy for prenatal prediction of ABO-HDFN. The available scientific literature suggests that the simultaneous use of Maternal sVE-cadherin and anti-A/B IgG titer is effective in preventing the disease. O Rh(D)- 515 pregnant women participated in the study. The levels of sVE-Cad and anti-A/anti-B antibodies in pregnant women in the third trimester are elevated in patients with ABO-HDFN [47]. There are also scientific studies analyzing the relationships between HDFN and additional biomarkers. A group of scientists used these markers: P-Selectin, Vascular Endothelial Cadherin, and Vascular Cell Adhesion Molecule-1. With the help of these biomarkers, prenatal screening, diagnosis, early prevention, and treatment of ABO-HDFN are possible [48]. Implementing similar combined approaches in relevant clinics provides a more effective prenatal diagnostic strategy for ABO-HDFN than using either marker alone.

Implementing molecular genotyping techniques may contribute to more accurate identification of blood group incompatibility and support the development of personalized strategies for early detection and prevention of HDFN complications in newborns.

Study limitations: The research study materials were collected from only one region of Georgia. The limited sample size does not allow us to draw a strong, definitive conclusion. Further large-scale multicenter studies are needed to validate these findings and to better define the genetic and immunological factors involved in the development of HDFN.

5 Conclusion

HDFN is prevalent in newborns with non-O blood groups, as evidenced by the relatively rare occurrence of the complete deletion c.261delG (homozygous) at SNP rs8176719 in HDFN. Non-O groups (A and B) were presented by (-; G) genotype in all cases. In ABO-HDFN, the highest percentage is represented by the O/A maternal/fetal phenotype. In the case of Rh incompatibility, it is Rh+/Rh-.

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APPENDIX

Table 1. Studied ABO-HDFN, Rh-HDFN, and unspecified-HDFN

HDFN types	N	%	95% CI (Wilson)	χ^2 (df=2)	P - value
ABO- HDFN	39	45.35	35.3-55.8%	6.44 (df=2)	0.04
Rh-HDFN	20	23.26	15.5-33.4%		
unspecified HDFN	27	31.4	22.5-41.9%		
Total	86	100			

Source: created by the authors.

Table 2. ABO/Rh phenotypic combinations in ABO-HDFN (2020-2024)

ABO/Rh phenotypic combinations	ABO-HDFN cases		
	N	%	95% CI (Wilson)
O(I),Rh ⁺	0	0.00	0.0-8.9%
O(I),Rh ⁻	0	0.00	0.0-8.9%
A(II),Rh ⁺	29	74.36	58.9-85.4%
A(II),Rh ⁻	2	5.13	1.4-16.9%
B(III),Rh ⁺	6	15.38	7.3-29.7%
B(III),Rh ⁻	1	2.56	0.05-13.2%
AB(IV),Rh ⁺	1	2.56	0.05-13.2%
AB(IV),Rh ⁻	0	0.00	0.0-8.9%
Total	39	100	

* χ^2 - 142.13, df = 7, p<0.001

Source: created by the authors.

Table 3. ABO/Rh phenotypic combinations in Rh-HDFN (2020-2024)

ABO/Rh phenotypic combinations	Rh-HDFN cases		
	N	%	95% CI (Wilson)
O(I),Rh ⁺	9	45.00	25.8-65.8%
O(I),Rh ⁻	0	0.00	0.0 -16.1 %
A(II),Rh ⁺	10	50.00	29.9-70.1%
A(II),Rh ⁻	0	0.00	0.0 -16.1 %
B(III),Rh ⁺	1	5.00	0.9 -23.6%
B(III),Rh ⁻	0	0.00	0.0 -16.1 %
AB(IV),Rh ⁺	0	0.00	0.0 -16.1 %
AB(IV),Rh ⁻	0	0.00	0.0 -16.1 %
Total	20	100	

* χ^2 -36.80, df=7, p<0.001

Source: created by the authors.

Table 5. Genotyping analysis in the studied newborns.

Genotype	Analysis	N	%	95% CI (Wilson)	χ^2 (df -2)
(-;-)	O Blood group	7	20.6	10.5-36.7%	34.6
(-;G)	A and B blood groups	27	79.4	63.3-89.5%	
(G;G)	A, B, and AB blood groups	0	0	0	

* χ^2 - 34.6, df=2, p < 0.0001

Source: created by the authors.

Table 6. Mother/Newborn group combinations for ABO-HDFN (2020-2024).

Newborn Mother	O(I) Rh ⁺	O(I) Rh ⁻	A(II) Rh ⁺	A(II) Rh ⁻	B(III) Rh ⁺	B(III) Rh ⁻	AB(IV) Rh ⁺	AB(IV) Rh ⁻
O(I),Rh ⁺			28	1	6	1	1	
O(I),Rh ⁻			1	1				
A(II),Rh ⁺								
A(II),Rh ⁻								
B(III),Rh ⁺								
B(III),Rh ⁻								
AB(IV),Rh ⁺								
AB(IV),Rh ⁻								
Total			29	2	6	1	1	

* A shaded box indicates the absence of the corresponding combinations in the mother/newborn.
Source: created by the authors.

Table 7. Mother/Newborn group combinations for Rh-HDFN (2020-2024).

Newborn Mother	O(I) Rh ⁺	O(I) Rh ⁻	A(II) Rh ⁺	A(II) Rh ⁻	B(III) Rh ⁺	B(III) Rh ⁻	AB(IV) Rh ⁺	AB(IV) Rh ⁻
O(I),Rh ⁺								
O(I),Rh ⁻	9		3					
A(II),Rh ⁺								
A(II),Rh ⁻			7					
B(III),Rh ⁺								
B(III),Rh ⁻					1			
AB(IV),Rh ⁺								
AB(IV),Rh ⁻								
Total	9		10		1			

* A shaded box indicates the absence of the corresponding combination in the mother/newborn.
Source: created by the authors.

Table 8. Distribution of O and non-O blood groups into different target groups.

Blood Group	Newborns with HDFN	newborns [19]	1-year-old infants [39]	Blood donors [40]	χ^2	P - value
O blood group	7 (20.6%; 95%CI:10.3-36.8)	91 (43.8%; 95% CI:37.2-50.5)	87 (43.1%; 95% CI:36.5-50.0)	373 (50.28%; 95% CI:46.6- 53.8)	14.7 138	0.002 1
Non- O (A, B, AB)	27 (79.4%; 95%CI 63.2-89.7)	117 (56.2%; 95% CI:49.5- 62.8)	115 (56.9%; 95% CI:50.0- 63.5)	370 (49.8%;95% CI: 46.2-54.4)		
Total	34	208	202	743		

Source: created by the authors.

Contribution of Individual Authors to the Creation of a Scientific Article (Ghostwriting Policy)

Marina Nagervadze: Conceptualization, study design, statistical analysis, genetic data interpretation, and manuscript revision. Marina Nagervadze was responsible for the statistical analysis of the study using standard biostatistical methods. She analyzed categorical variables by calculating frequencies, percentages, and 95% confidence intervals using the Wilson score method. She performed chi-square (χ^2) tests to evaluate differences between categorical variables and conducted chi-square goodness-of-fit analyses to assess whether the observed genotype and phenotype frequencies significantly differed from the expected distributions.

Leila Akhvlediani: Immunohematological investigation and laboratory diagnostics. Leila Akhvlediani was responsible for the immunohematological analyses of hemolytic disease of the fetus and newborn (HDFN). She performed ABO and Rh blood group determination using monoclonal anti-A, anti-B, and anti-D antibodies, conducted direct and indirect Coombs tests to confirm immune-mediated HDFN, and contributed to the immunological evaluation and interpretation of serological findings related to maternal–fetal blood group incompatibility. She also participated in the validation and interpretation of the immunohematological laboratory results.

Shorena Gabaidze and Rusudan Khukhunaishvili: Molecular investigation and laboratory analyses. Shorena Gabaidze performed the molecular analyses, including genotyping procedures and analysis of the SNP rs8176719 for molecular characterization of the ABO blood group system. They also contributed to the interpretation of the genetic findings, including the analysis of the ABO gene polymorphism (rs8176719) used for the detection of the 261G deletion in exon 6 and identification of the ABO O allele. They participated in the critical revision of the manuscript.

Tamar Bakhtadze: Clinical investigation, neonatal assessment, and data interpretation. Tamar Bakhtadze analyzed the laboratory findings of newborns with hemolytic disease of the fetus and newborn (HDFN), including total serum bilirubin and other indicators of hemolysis. She evaluated the clinical signs and severity of HDFN, contributed to the clinical diagnosis of affected newborns, and participated in interpreting the clinical and laboratory data.

Jumber Ungiadze and Nato Gorgadze: Clinical investigation, neonatal assessment, and data interpretation. Nato Gorgadze analyzed laboratory results from newborns with HDFN, including total serum bilirubin and other hemolysis markers. She assessed the clinical manifestations of HDFN, contributed to establishing the clinical diagnosis, and participated in the interpretation and validation of the clinical findings.

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Conflict of interest

The authors have no conflicts of interest.

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